Supporting Information

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SI Methods

Animals. Timed pregnant female C57BL/6 mice were obtained from Charles River Laboratories. Sciatic nerves were obtained from postnatal day 3 to 5 WT C57BL/6 mice and cultured as described later. A colony of 32KO mice was bred in-house from Cx32-null founders (provided by S. Bennett, Ottawa, ON, Canada) that had been back-crossed at least 15 generations into C57BL/6 background. All animals were provided food and water ad libitum and housed according to the Guide for the Care and Use of Laboratory Animals.

SC Cultures. SC cultures were established using a combination of standard methods, with some modifications (1–3). Briefly, sciatic nerves from postnatal day 3 to 5 neonatal mice were dissected and incubated in collagenase (2 mg/mL) and trypsin (0.125%) for 30 min at 37 °C. The nerves were transferred to $B27NBM_A$ [Neural Basal A Medium (Invitrogen); 0.5 mM GlutaMax (Invitrogen); 10 mM Hepes, pH 7.4; B27 supplement (Invitrogen); and Primocin (InvivoGen)] supplemented with 10% bovine growth serum (HyClone). The nerves were gently dissociated with a Pasteur pipet and filtered through a 40-μm nylon filter (Falcon). The dissociated SCs were seeded onto polyD-lysine-coated dishes at 0.23 sciatic nerve equivalents/cm^2 in $B27NBM_A$ with 10% bovine growth serum and supplemented with recombinant human GGF2 (5 ng/mL; gift from Cambridge Neurosciences/Acorda) and forskolin (10 μM). Eighteen to 24 h after plating, cultures were switched to serum-free $B27NBM_A$ supplemented with GGF2 and forskolin. GGF2 and forskolin supplements were withdrawn when the cultures reached approximately 90% confluence at 4 to 5 DIV and maintained in $B27NBM_A$ alone thereafter. GGF2 and forskolin supplementation was discontinued at least 24 h before any experimental treatment. The cultures are >99.5% pure by 2 days in culture as determined by morphology and immunostaining for p75NTR, S100, and CNPase.

For immunofluorescence, BrdU incorporation, and electrophysiology studies, SCs were plated onto polyD-lysine-treated no. 0 glass coverslips. For Alamar Blue studies, SCs were seeded into polyD-lysine-treated, clear-bottom 96-well plates (Costar).

Immunocytochemistry. Cultures were fixed at room temperature with Histochoice (Amresco), permeabilized, and blocked with ICC block (PBS, 2% BSA, 2% goat serum, 0.05% Tween-20, 0.1% fish gelatin, 0.1% Triton X-100). Coverslips were incubated in Image-IT (Invitrogen) and processed for immunofluorescence using standard techniques. Signal was detected with Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and the coverslips were mounted using ProLong Gold plus DAPI (Molecular Probes). Digital images were collected using a Magnafire advanced imaging system (Olympus) and appropriate filters. Except where noted, images were obtained at magnification ×400. All images for a given data set were collected and processed in an identical manner. For each experiment, images from 3 to 4 random fields were collected for each coverslip, with at least 3 replicate coverslips per experimental group. Experiments were repeated at least 3 times. Values from cell-free areas were used to determine background for each image. Integrated fluorescence values were obtained from background-subtracted images using the cell scoring and multi-wavelength cell scoring applets in Metamorph 7 image analysis software (Molecular Devices). Thresholds were determined using negative control images, and cellular integrated fluorescence for these images constituted less than 10% of positively scored cells. "Hoechst" refers to Hoechst 33342 nuclear stain.

Antibodies. Primary antibodies were used at the following dilutions for immunocytochemistry: Cx32 (7C6.7C monoclonal or EL1 affinity purified polyclonal; gifts from E. Hertzberg, New York, NY) 1:250; p75NTR (Chemicon) 1:1,000; erbB3 (Santa Cruz Biotechnology) 1:100; erbB2 (LabVision) 1:1,000; and BrdU (LabVision) 1:150. Alexa Fluor-tagged secondary antibodies (Molecular Probes) were diluted 1:400. Primary antibody dilutions for Western blot were increased 5 to 10 fold depending on the antibody. HRP-tagged secondary antibodies (Pierce) were used at 15 to 30 ng/mL for Western blots. Figs. S5 and S6 demonstrate antibody specificity for Cx32 antibodies used in Western blot and immunofluorescence studies.

Proliferation and Survival Assays. ***BrdU Incorporation:*** Cultures were treated with BrdU (10 μM; Molecular Probes) overnight in the presence or absence of GGF2 and fixed as described earlier. The samples were denatured 30 min with 2N HCl in 0.1% Triton X-100, neutralized with 0.2 M borate buffer, and washed with PBS 0.95% Tween solution. The samples were then processed for BrdU immunofluorescence as described. Immunofluorescent detection of Cx32 preceded denaturation and immunolabeling for BrdU.

Alamar Blue: Cultures were seeded onto black-wall, clear-bottom 96-well plates (Costar/Corning). Alamar Blue (Invitrogen/Biosource) (4–6) was added to a final concentration of 10% (vol/vol) along with GGF2 and buffer treatments. Alamar Blue fluorescence was read with a Flexstation II microplate reader (Molecular Devices) using the manufacturer's suggested settings at 0.5, 16, 24, and 48 h after addition of Alamar Blue and treatment with GGF2 or buffer.

Western Blot. SCs were washed on ice with PBS solution and harvested in modified RIPA buffer with 1% lithium dodecyl sulfate supplemented with freshly added Complete protease inhibitor (Roche). Cleared supernatants were divided into aliquots and stored at -20 °C. Total proteins were determined using a CB-X protein assay (GBiosciences). The samples were diluted with NuPage sample buffer, and β-mercaptoethanol was added to a final concentration of 5%. The samples were denatured for 30 min at room temperature to limit multimer formation. Forty micrograms total protein was loaded for each sample and separated on 4% to 12% NuPage gel (Invitrogen). The gels were transferred to PVDF by semi-dry electroblot, processed for Western blot, and developed using chemiluminescence detection (Pierce). Films were scanned on an Agfa flatbed scanner and the resulting images were analyzed using ImageJ. Western blots experiments were repeated at least 2 times.

Electrophysiology. Dual whole-cell patch clamping was performed as previously described (7). Recording solutions were as follows: (*i*) pipette solution, 145 mM CsCl, 5 mM EGTA, 0.5 mM $CaCl_2$, 10.0 mM Hepes, pH 7.2; (*ii*) standard bath solution, 150 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM dextrose, 2 mM pyruvate, 10 mM Hepes, pH 7.4.

Additional Reagents. Forskolin (Sigma); myristolated cell-permeant PKA inhibitor (PKI 14–22 Amide; Calbiochem); H8 (Calbiochem); rhGGF2 (Cambridge Neuroscience/Acorda); collagenase type I (Sigma); trypsin (Invitrogen); polyD-lysine

(70–150,000 kDa; Sigma); normal goat serum (Sigma); and fish gelatin (Sigma).

Statistical Analyses. Raw data were assembled and imported into GraphPad 4.03 or Instat 3.06 (GraphPad) software for statistical analyses. Data are displayed in bar graphs as mean values with error bars denoting SEM. The Fisher exact test, *t* test, or ANOVA with Newman-Keuls post-test were used where appropriate to determine statistical significance.

Supporting Experiments

GGF2 Concentration Response Relation. SC cultures were routinely treated with 5 ng/mL GGF2, which corresponds to approximately 5 times the EC_{50} (1.13 ng/mL) for the proliferative effect of GGF2 preparation on rat SCs, as reported by the supplier (Acorda/Cambridge Neurosciences). Cultured (mouse) WT SCs were treated with increasing concentrations of GGF2 and assayed for Cx32 to establish a reasonable working concentration. Both normalized Cx32 immunofluoresce labeling (Fig. S3*B*) and Western blot analysis (Fig. S3*A Inset*) demonstrated a readily observable response at 5 ng/mL GGF2 with no response at 0.5 ng/mL and a decreased response at 20 ng/mL, consistent with desensitization. (There is another concentration response relation in (Fig. S7*C*).

Antibody Specificity. Little or no staining was detected in immunolabeled 32KO SCs, confirming the specificity of the affinity purified polyclonal anti-Cx32 antibody (EL1; a gift from E. Hertzberg) used in these studies (Fig. S5). The specificity of 7c6.7c, a mouse monoclonal anti-Cx32 (a gift from E. Hertzberg, Bronx, NY) was confirmed by Western blot analysis using WT and 32KO tissues and immunoprecipitation from lysates of cultured SCs (Fig. S6).

1. Porter S, Clark MB, Glaser L, Bunge RP (1986) Schwann cells stimulated to proliferate in the absence of neurons retain full functional capability. *J Neurosci* 6:3070–3078.
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3. Brockes JP, Fryxell KJ, Lemke GE (1981) Studies on cultured Schwann cells: the induction of myelin synthesis, and the control of their proliferation by a new growth factor. *J Exper Biol* 95:215–230.
4. Nikolaychik VV, Samet MM, Lelkes PI (1996) A new method for continual quantitation of viable cells on endothelialized polyurethanes. *J Mater Sci* 7:881–891.
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7. Abrams CK, *et al.* (2003) Pathogenesis of X-linked Charcot-Marie-Tooth disease: differential effects of two mutations in connexin 32. *J Neurosci* 23:10548–10558.

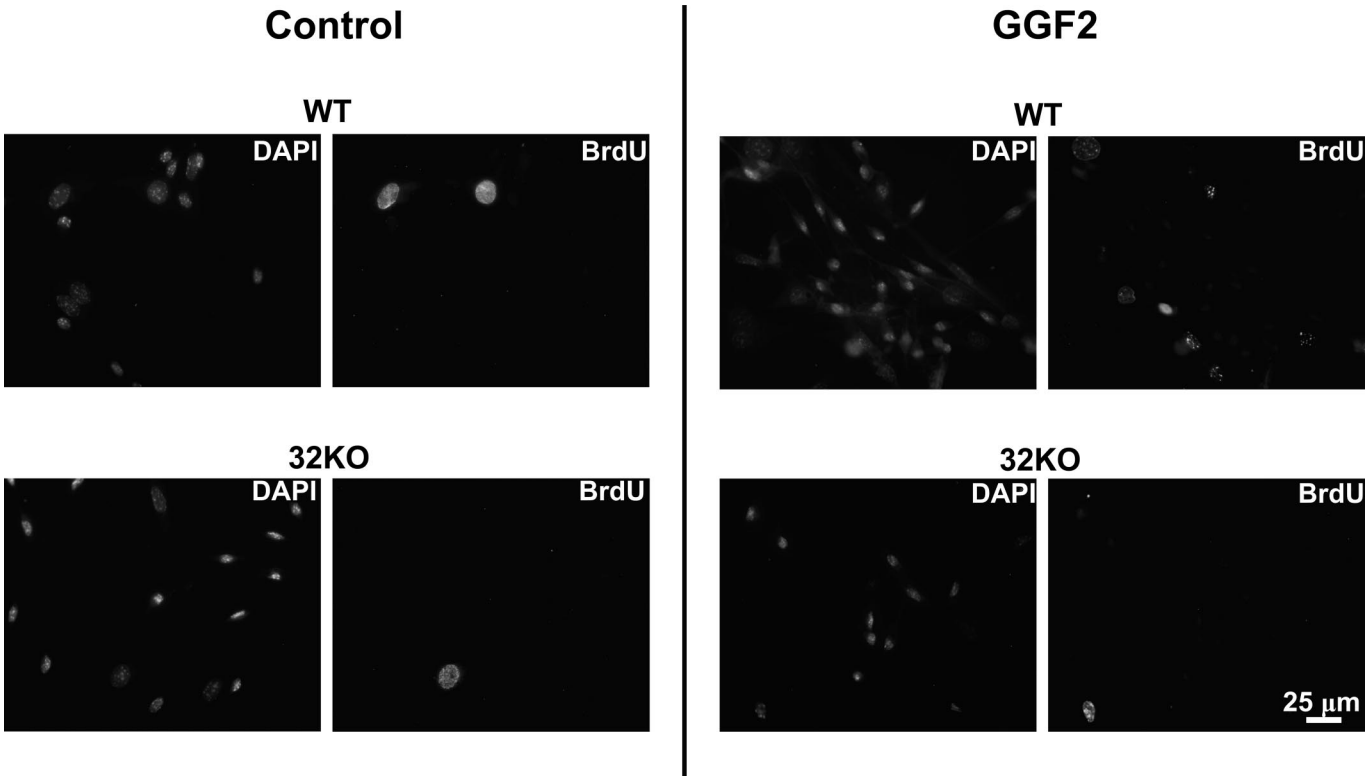
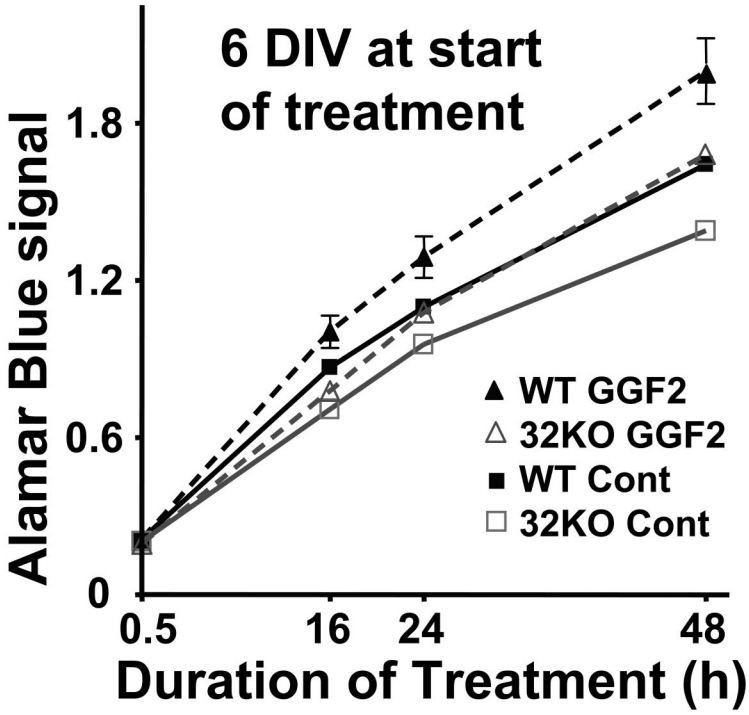


Fig. S1. Incorporation of BrdU is greater in control and GGF2-treated WT SCs than in control and GGF2-treated 32KO SCs. WT and 32KO cultured SCs at 6 DIV were incubated with BrdU (10 μM) overnight in the presence or absence of GGF2 (5 ng/mL) and fixed the next day as described in *SI Methods*. BrdU-positive nuclei tended to be larger than BrdU-negative nuclei.



Time h	WT Cont vs WT GGF2	32KO Cont vs 32KO GGF2	WT Cont vs 32KO Cont	WT GGF vs 32KO GGF
0.5h	ns	ns	ns	ns
16h	ns	ns	ns	P<0.01
24h	P < 0.05	ns	ns	P<0.01
48h	P<0.001	P<0.001	P<0.001	P<0.001

Fig. S2. WT SCs show greater rates of generation of Alamar Blue and more stimulation by GGF2 than 32KO SCs. WT and 32KO SCs were seeded onto 96-well plates as described in *Methods*. Fresh B27NBM$_A$ containing Alamar Blue precursor (10% vol/vol) with or without GGF2 (5 ng/mL) was added to the cultures at 6 DIV. Alamar Blue fluorescence was measured at 0.5, 16, 24, and 48 h after treatment (6–8 DIV). Alamar Blue fluorescence is proportional to cell number and metabolic activity and is an index of cell viability and proliferation (4–6). The table summarizes significance testing by ANOVA. (No. of replicate wells: $n = 24$.)

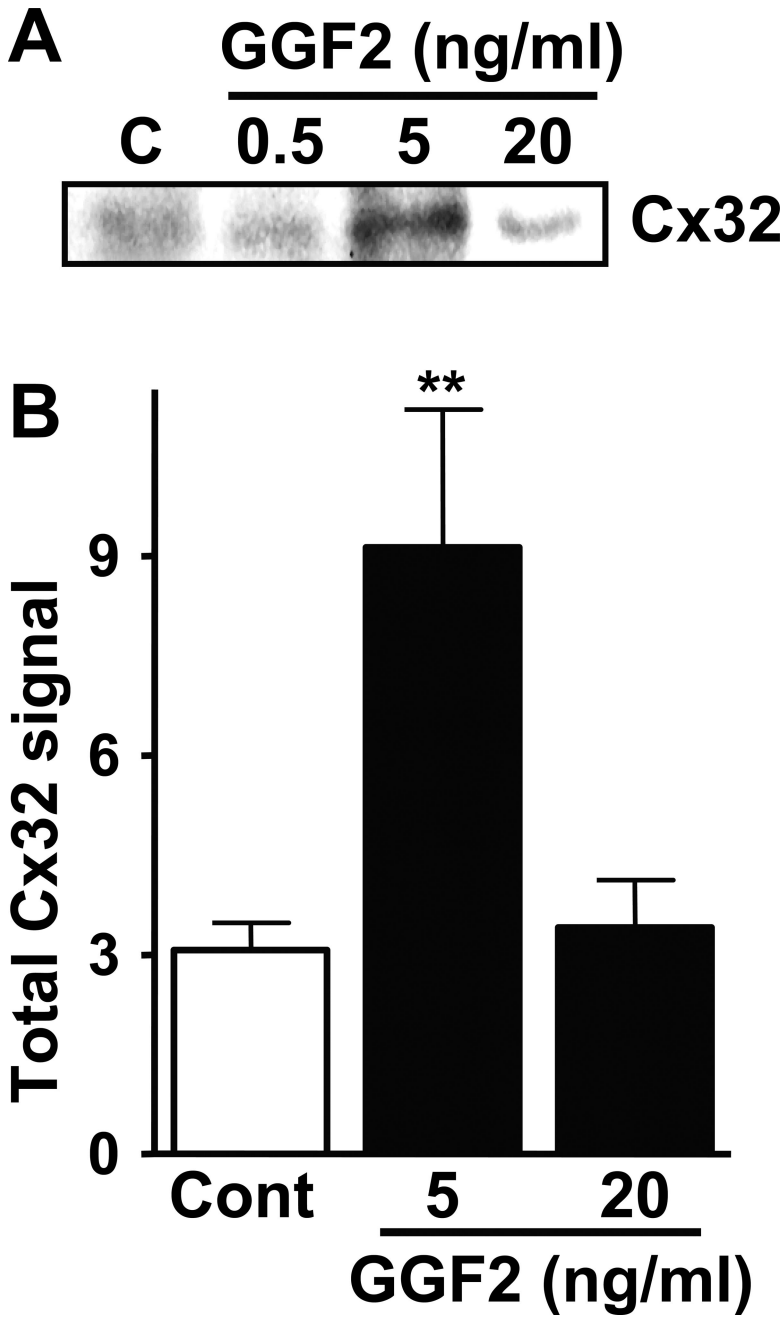


Fig. S3. GGF2 increases SC expression of Cx32 in cultured SCs in a concentration-dependent manner. (*A*) Cultured WT SCs (after 9 DIV) were treated overnight with 0, 0.5, 5, or 20 ng/mL GGF2 and harvested for Western blot analysis. The blots were probed with monoclonal anti-Cx32 (7c6.7c) and were developed using chemiluminescent detection. There was a readily detectable response at 5 ng/mL and no response above control at 0.5 or 20 ng/mL. The figure represents 2 experiments. (*B*) WT SCs were seeded into 96-well plates and, at 9 DIV, were treated overnight with 0, 5, or 20 ng/mL GGF2 and immuno-labeled for Cx32 using affinity purified polyclonal antibody (EL1) and counterstained with Hoechst stain. Total Cx32 and Hoechst fluorescence signals were measured in a Flexstation II microplate reader (Molecular Devices). The Cx32 signal was normalized to the Hoechst signal and expressed in arbitrary units. (No. of wells per group: $n = 10$; **, $P < 0.01$.)

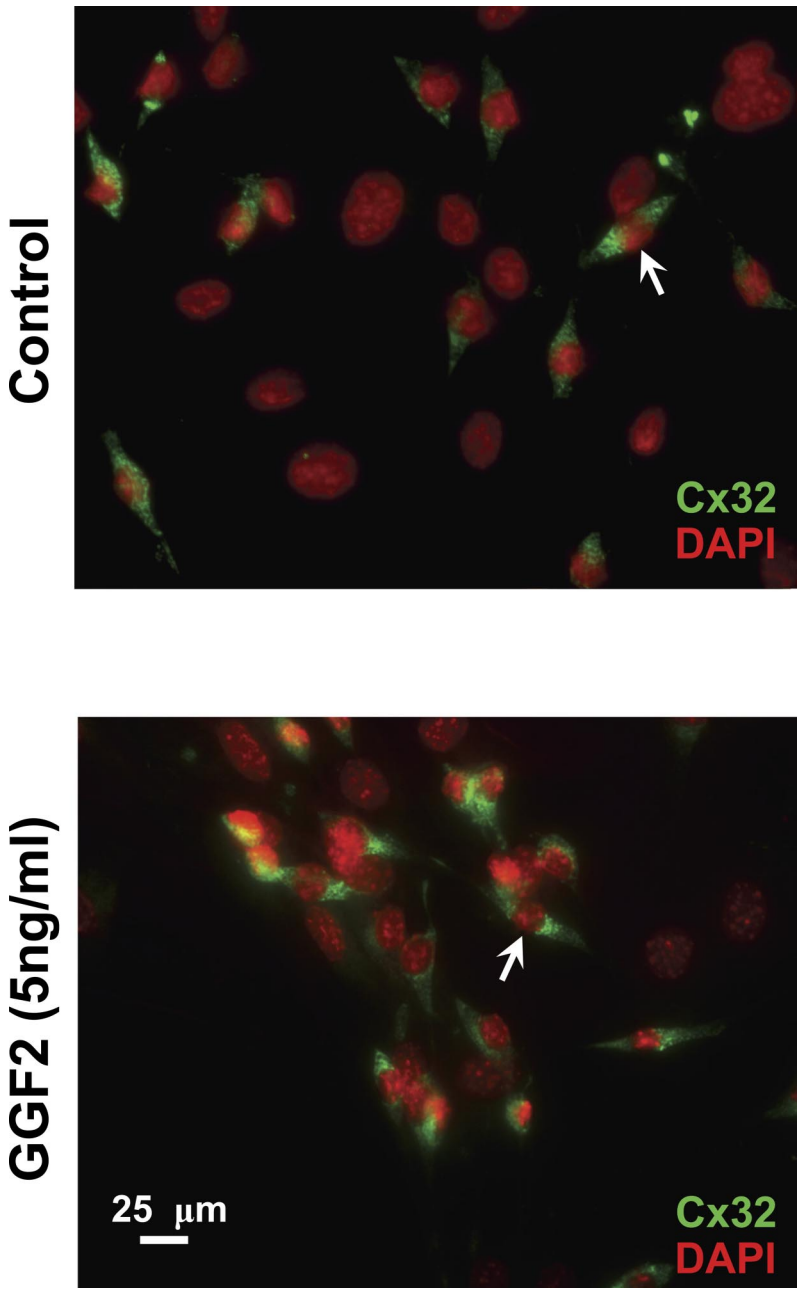


Fig. S4. GGF2 increases expression of Cx32 in cultured SCs. WT SCs were plated on coverslips and, at 8 DIV, were treated overnight in control medium (*Upper*) or GGF2 (5 ng/mL, *Lower*). Cells were fixed and immuno-labeled with affinity purified polyclonal antibody to Cx32 (EL1; green) and counterstained with DAPI (red). The majority of SCs are spindle-shaped with the ovoid nucleus nearly filling the maximum cell diameter. Cx32 labeling was concentrated in the cytoplasm, and could form 2 triangular regions on either side of the nucleus (arrows). (Calibration bar: 25 μm.)

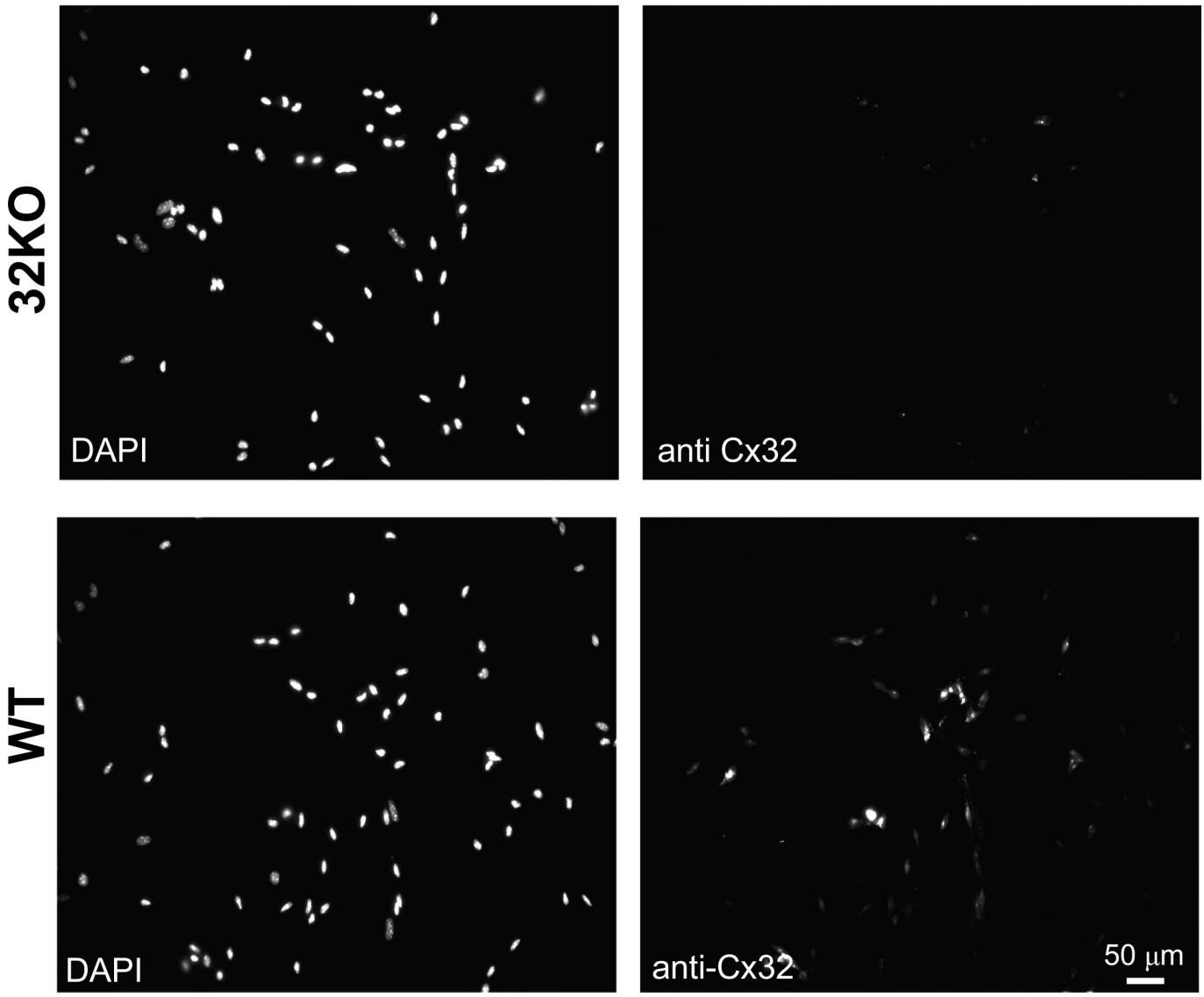


Fig. S5. Immunolabeling of WT and 32KO cultured SCs demonstrates the specificity of the rabbit anti-Cx32 antibody used for the immunofluorescence studies in this study. WT and 32KO SCs were seeded onto polyD-lysine coated coverslips. Forty-eight hours after plating, the cells were fixed and processed for immunofluorescence using affinity-purified rabbit anti-Cx32 (EL1, 1:250) and mounted using Prolong Gold with DAPI (Molecular Probes). There was little or no labeling of 32KO cultures and extensive labeling of WT cultures. Similar results were seen in images from 3 replicates from each group. (Calibration bar: 50 μm.)

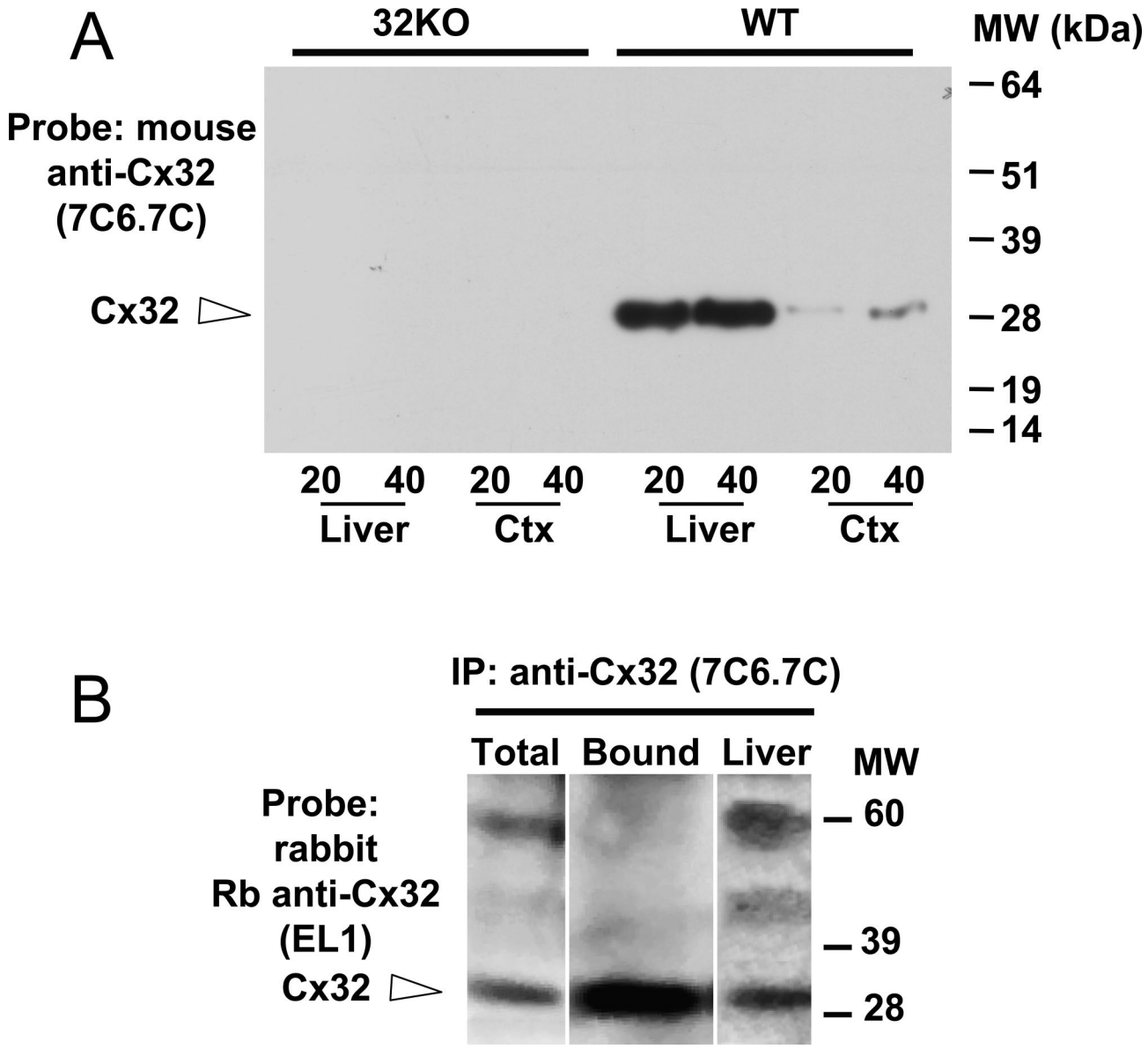


Fig. S6. Western blot analysis and immunoprecipitation demonstrate the specificity of the monoclonal and polyclonal Cx32 antibodies used in these studies. (*A*) Liver and cortex (*Ctx*) were harvested from 6-week-old adult WT and 32KO mice. Twenty and 40 μg of extract from each sample were loaded and separated on a 4% to 12% NuPage TrisBis gel (Invitrogen) and transferred and processed for Western blot using monoclonal mouse anti-Cx32 (7c6.7c, 1:2,000) and WestPico (Pierce) chemiluminescence detection. (*B*) Control-, GGF2-, and forskolin-treated cultured SCs were harvested for immunoprecipitation. Briefly, cells were harvested on ice in half-volume RIPA/LDS-1 with Complete protease inhibitor, and aliquots of total extract were taken. Following lysis, the samples were diluted with 2 volumes RIPA without LDS and pre-cleared with protein A/G Sepharose (Santa Cruz Biotechnology) at room temperature, and the supernatant was incubated overnight at 4 °C with monoclonal anti-Cx32 (7c6.7c). Protein A/G Sepharose was added and the sample incubated further at room temperature. The beads were washed and eluted with IgG Elution buffer (Pierce) as recommended in the Seize Classic Immunoprecipitation protocol (Pierce). Total SC extract (25 μg), eluted immunoprecipitation samples, and liver extract (10 μg, Santa Cruz Biotechnology) were loaded and separated on a 10% NuPage gel. The gel was transferred and processed for Western blot using affinity-purified rabbit anti-Cx32 (EL1, 1:1000) and WestDura (Pierce) chemiluminescence detection. Immunoprecipitation resulted in significant recovery of the anti-Cx32 bound fraction.

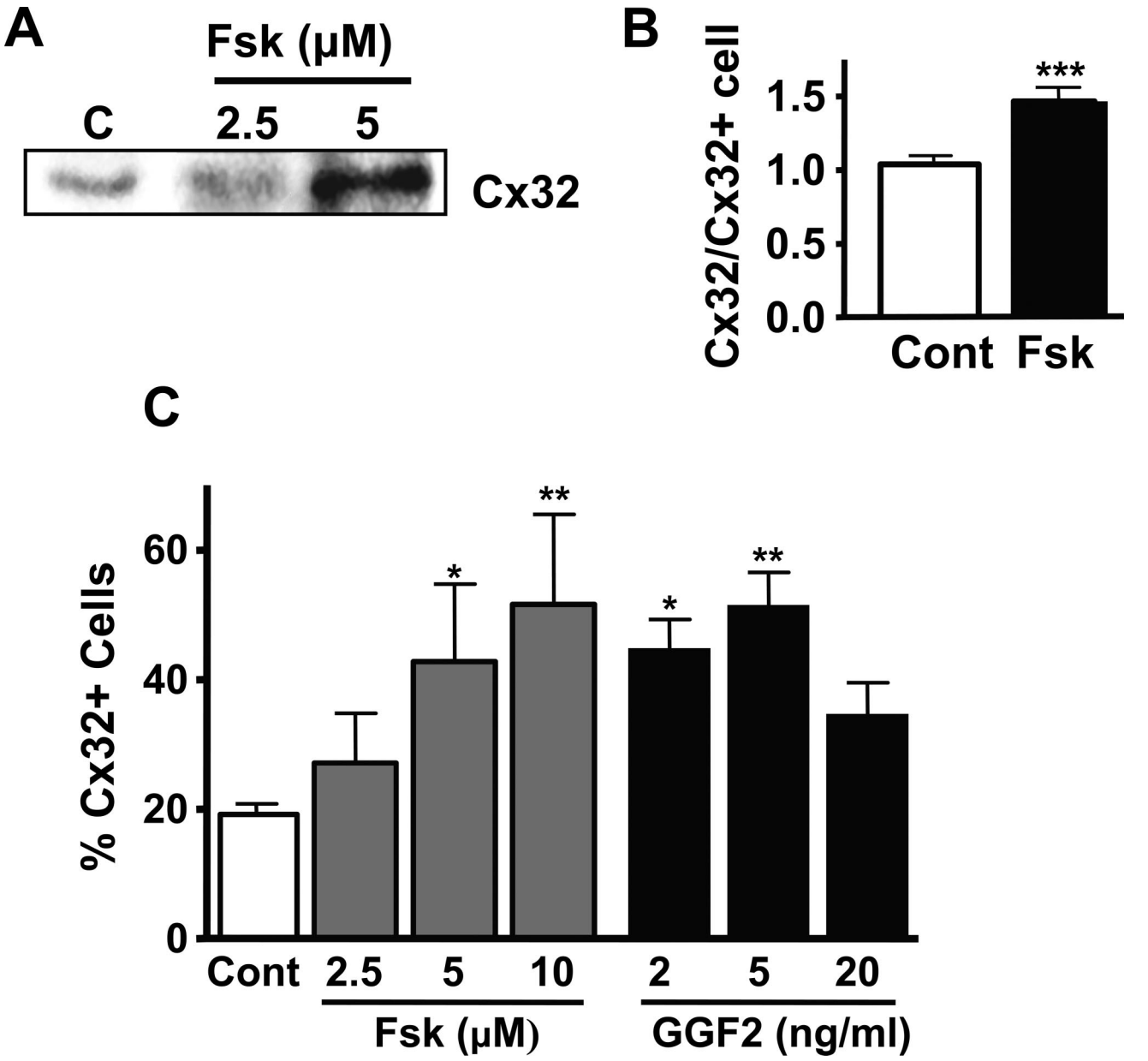


Fig. S7. Forskolin increases expression of Cx32 by SCs. (*A*) Cultured WT SCs at 8 DIV treated overnight with 0, 2.5, or 5 μM forskolin (*Fsk*) were processed for Western blot and probed with monoclonal anti-Cx32 (7c6.7c) as described. Figure represents 2 two blots. (*B*) WT SCs at 8 DIV treated overnight with vehicle or forskolin (10 μM) were immuno-labeled with affinity-purified polyclonal antibody to Cx32 (EL1) and counterstained with DAPI. (No. of cells: control, $n = 508$; Fsk, $n = 330$; ***, $P < 0.001$.) Forskolin treatment increased the amount of Cx32 per Cx32-positive cell. (*C*) The percentage of cells that were Cx32-positive was increased by treatment with increasing concentrations of forskolin (at 8 DIV). GGF2 also increased the percentage of cells that were Cx32-positive at concentrations similar to those that were effective in increasing total Cx32 (Fig. S3) treatment. (No. of images: control, $n = 30$; 2.5 μM forskolin, $n = 14$; 5 μM forskolin, $n = 10$; 10 μM forskolin, $n = 9$; for all concentrations of GGF2, $n = 4$; *, $P < 0.05$, **, $P < 0.01$.)

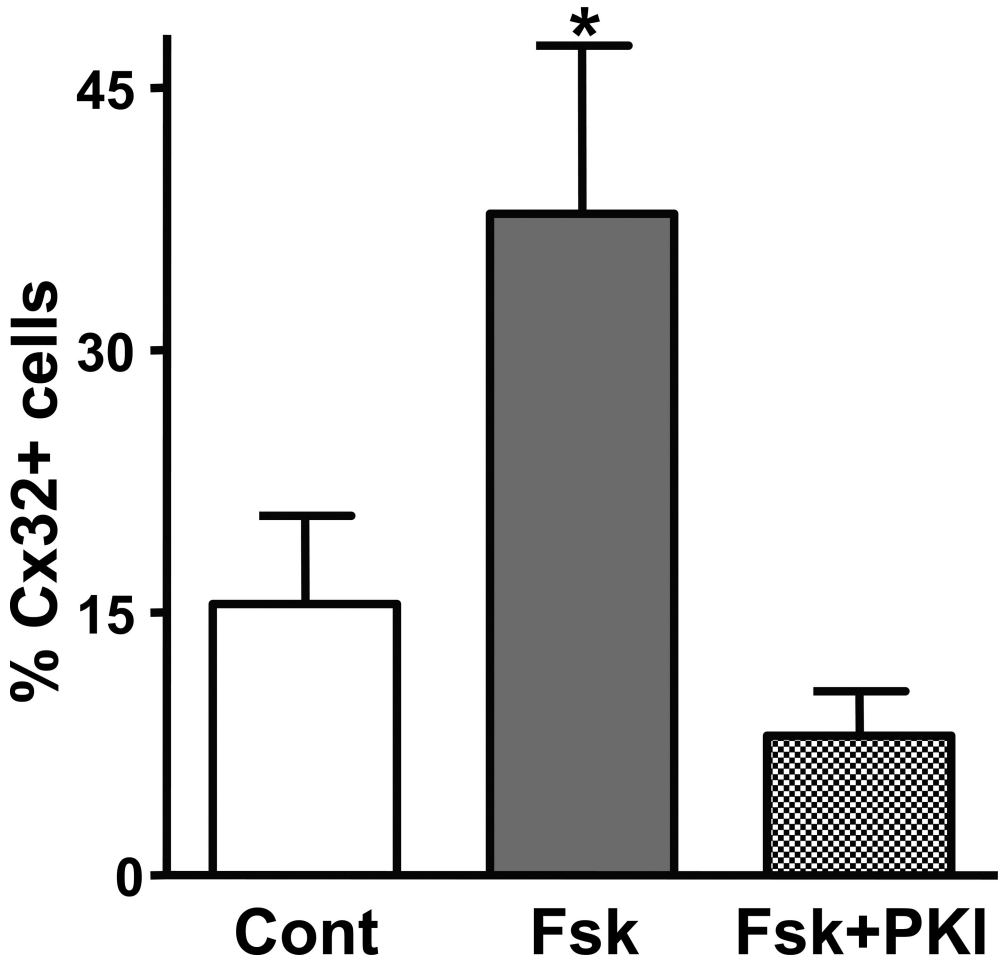


Fig. S8. Treatment with PKAInh (*PKI*) suppresses forskolin (*Fsk*)-mediated increase in percentage of Cx32-positive SCs. Cultured SCs maintained for 8 DIV in $B27NBM_A$ were treated overnight with forskolin (10 μM) in the presence or absence of PKAInh (350 nM; K_i = 35 nM) and immuno-labeled with a polyclonal antibody against Cx32 (EL1). Positive cells were scored and normalized to total cells counts per well. (No. of replicate wells analyzed: control, $n = 16$; Fsk, $n = 13$; Fsk + PKI, $n = 8$; *, $P < 0.05$.)